REMARKS

Claim Amendments

Claims 43, 44, 46-48, and 90-91 have been amended. Claims 40-42 have been canceled without prejudice or disclaimer to the subject matter therein. Claims 94-97 have been added. Upon entry of the foregoing amendment, claims 43-48 and 88-97 are pending in the application.

Claim 43 has been amended to recite that the at least one bacteriophage be applied "before transferring said at least one freshly-hatched bird to a chicken house." Support for this amendment may be found, for example, on page 39, lines 18-26.[1]

Claims Claim 44 and 46-48 have been amended to correct minor, typographical errors.

Claim 46 has also been amended to recite that the drinking water containing the at least one bacteriophage is provided "to poultry." Support for this amendment may be found, for example, on page 17, lines 1-4 and page 39, lines 27-28.

Claims 90-91 have been amended to recite that the at least one bacteriophage is sprayed onto the surface of the eggs. Support for this amendment may be found, for example, on page 39, lines 1-10 and page 40, lines 15-16.

Support for new claims 94-95 may be found, for example, on page 39, lines 27-28. Support for new claim 96 may be found, for example, on page 31, lines 18-19, page 39, lines 15-17 and page 35, lines 21-23. Support for new claim 97 may be found, for example, on page 17, lines 5-7.

Applicants respectfully request entry of the foregoing above amendment and submit that the above amendment does not constitute new matter.

Statement of Substance of Interview Under 37 C.F.R. § 1.133(b)

In accordance with 37 C.F.R. § 1.133(b) and M.P.E.P. § 713.04, Applicants herein provide a summary of the interview held on August 2, 2007 with Examiners Stacey B. Chen and Nicole E. Kinsey, Ph.D. ("interview"). Applicants thank Examiners Chen and Kinsey for agreeing to conduct the interview and appreciate the courtesies extended by the Examiners.

During the interview, the parties discussed the rejections set forth in the Office Action and in particular, the Taylor *et al.* (U.S. Patent No. 2,851,006, hereinafter "Taylor"), Day *et al.* (U.S.

[1] The citations referred to herein are taken from the substitute specification filed on May 28, 2004.

Patent No. 4,851,240, hereinafter "Day"), and Merril, *et al.* (U.S. Patent No. 5,811,093, hereinafter "Merril") references.

Applicants argued that Taylor is limited to methods of introducing bacteriophage into an egg for the purpose of increasing the percentage of hatched birds and does not teach applying bacteriophage onto only the exterior surface of an egg. Applicants also argued, and the Examiners agreed, that Taylor does not teach "spraying" bacteriophage onto the surface of eggs. In the amendment provided herein, Applicants have amended independent claim 90 to require the limitation of "spraying."

Applicants also argued that Merril is directed to a method of bacteriophage therapy in animals (e.g., livestock) comprising administering a genetically engineered bacteriophage and does not teach applying bacteriophage to "freshly-hatched birds." Applicants explained that at the time of hatch, a bird's digestive tract is usually sterile and is not infected with pathogenic bacteria (e.g., *Salmonella*). Applicants further explained that a bird is typically transferred to a chicken house within twenty-four hours after hatching. Applicants indicated that during this time — after hatching and before transferring the bird to the chicken house — at least one bacteriophage is applied to the bird in accordance with the claimed invention. Applicants reiterated that Merril does not teach or suggest such a method. In particular, Applicants pointed out that Merril relates to "therapy," whereas in the claimed invention, the "freshly-hatched" birds are not infected and therefore do not require "therapy." Applicants have amended independent claim 46 to recite that the at least one bacteriophage be applied "before transferring said at least one freshly-hatched bird to a chicken house."

Applicants further argued that Day is directed to a method of controlling bacterial growth in fermented animal foodstocks, especially silage (preserved forage) for feeding ruminant livestock and does not relate to poultry or poultry processing. Applicants pointed out that "livestock" and "poultry" are different classes of animals and that when Day refers to "livestock," it is not intended to include "poultry." Applicants indicated they would reiterate their arguments with supporting documentation in its next response. Applicants have amended independent claim 43 to recite that the drinking water containing the at least one bacteriophage is provided to "poultry."

Rejections Under 35 U.S.C. §102(b)

Claims 40-42 and 90-93 stand rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by Taylor.

Applicants respectfully traverse this rejection.

Initially, Applicants submit the rejection with respect to claims 40-42 is *moot* as these claims have been canceled. Applicants also submit that Taylor does not teach the claimed method, as amended, because Taylor does not teach the step of "spraying" at least one bacteriophage onto the surface of eggs. Accordingly, Applicants respectfully request withdrawal of the 102(b) rejection over Taylor.

Claims 46-48 stand rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by Day.

The Office Action states that Day teaches administering water containing bacteriophages to livestock. The Office Action also states that "chickens are livestock." *See* O.A. at pages 3-4.

Applicants respectfully disagree and traverse this rejection.

Initially, Applicants submit that "livestock" does not necessarily include "poultry" or "chickens." Indeed, the United States Code in at least one instance separately defines "livestock" and "poultry." *See* 7 U.S.C. §§ 182 (4) and (5) ("The term 'livestock' means cattle, sheep, swine, horses, mules, or goats…"; "The term 'poultry' means chickens, turkeys, ducks, geese, and other domestic fowl."), attached herewith as **Exhibit A**. Accordingly, "chickens" are not necessarily "livestock" as the Office Action contends.

Applicants also submit that Day relates to methods of controlling the microbial flora in ruminant livestock. Day teaches that silage is an important part of ruminant livestock feeding regimens in the economic production of milk and meat by farmers. *See* col. 1, lines 31-37. Silage is prone to secondary fermentation by spoilage microorganisms (e.g., the proliferation of *Clostridium* species). *See* col. 1, lines 55-58. Secondary fermentation causes the breakdown of sugars to butyric acid and ammonia, thereby significantly reducing the nutritional value of the silage and reducing edibility. *See* col. 1, lines 58-61. To prevent such secondary fermentation, Day proposes adding bacteriophages (e.g., *Clostridium*-specific phages) to silage. *See e.g.*, col. 4, lines 10-17.[2]

Specifically, Day states that adding such bacteriophage to silage has beneficial effects to ruminant livestock:

[2] The art previously recognized that the manipulation of ammonia levels with an agent such as urea shift populations of bacteria in rumen beneficially to promote digestion of barley. *See* Wallace, R.J. "Effect of Ammonia Concentration on the Composition, Hydrolytic Activity and Nitrogen Metabolism of the Microbial Flora of the Rumen," J. of Applied Bacteriology 46: 443-455 (1979), attached herewith as **Exhibit B**.

> ...silage treated with *Clostridium*-specific phage is not only preserved from clostridial degradation, but also transmits the phage to the recipient ruminant. This destroys the clostridial flora in the rumen, allowing the animal to make more efficient use of its food. Col. 4, lines 35-40.

Day also suggests that "a use or process as described above is effective not only as a food preservative but also as a growth promoter for livestock, by causing more efficient utilisation of nutrients." Col. 4, lines 43-46.

Consistent with the focus on ruminants, Day discusses the use of bacteriophage in drinking water for ruminant livestock, stating:

> ***Addition of specific clostridial phage 10^6-10^7 to drinking water*** and/or feedingstuffs ***will control butyric acid metabolism in the rumen***, leading to useful weight gain (and milk production ***in cattle***)." (emphasis added) Col. 4, lines 47-50.

The only other instance where Day specifically discusses administering drinking water containing bacteriophage to livestock is in Example 8. *See* col. 9, lines 15-52 (administering drinking water containing *Clostridium*-specific bacteriophages to ***cows***). (emphasis added)

Poultry are a class of farm animals distinct from the ruminant livestock addressed by Day. Applicants submit that Day does not relate to poultry or methods of poultry processing. Rather, Day relates to adding specific bacteriophage preparations to silage and/or drinking water for consumption by ruminant livestock. Indeed, in every instance that Day specifically discusses administering drinking water containing bacteriophage, it is clear that Day is referring to ruminant livestock (e.g., cattle) and not poultry (e.g., chickens). *See e.g.*, col. 4, lines 47-58 and Example 8. Accordingly, Applicants submit that because Day is limited to methods of providing drinking water containing specific bacteriophages to ruminant livestock, Day does not teach or suggest the claimed invention.[3]

In view of the foregoing, Applicants respectfully request withdrawal of the 102(b) rejection over Day.

[3] Applicants submit that new claim 97, directed to a method of poultry processing comprising providing food containing at least one bacteriophage to poultry, is patentable over Day for the same reasons as discussed above for claims 46-48.

Rejections Under 35 U.S.C. § 103(a)

Claims 43-45 and 88-89 stand rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Merril, in view of Taylor and Holzman (Genetic Engineering News, hereinafter "Holzman").

Applicants respectfully traverse this rejection.

Initially, Applicants note that claim 43 has been amended to recite that the at least one bacteriophage be applied "before transferring said at least one freshly-hatched bird to a chicken house." During the interview, Applicants explained that at the time of hatch, a bird's digestive tract is usually sterile and the bird is not infected by pathogenic bacteria.[4] Applicants also explained that a bird is typically transferred to a chicken house within twenty-four hours after hatching.[5] Applicants indicated that during this time — after hatching and before transferring the bird to the chicken house — at least one bacteriophage is applied to the bird in accordance with the claimed invention.

Applicants submit that the references alone, or in combination, do not teach each and every element of the claimed invention, and therefore do not render the claimed invention obvious.

Merril relates to a general method of bacteriophage therapy in animals comprising administering a genetically engineered bacteriophage capable of delaying inactivation by an animal's host defense system.[6] Indeed, Merril is limited to a method of treating animals (in general) by administering a specific type of bacteriophage via any standard route of administration for the purpose of prolonging phage viability in the body. As discussed during the interview, Merril relates to "therapy," whereas in the claimed invention, the "freshly-hatched" birds are birds that are not infected by pathogenic bacteria and therefore do not require "therapy." Accordingly, because Merril

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[4] *See e.g.*, Amit-Romach et al., "Microflora Ecology of the Chicken Intestine Using 16S Ribosomal DNA Primers," Poultry Science, 83:1093-1098 (2004), attached herewith as **Exhibit C**.

[5] *See e.g.*, Donald D. Bell et al. Commercial Chicken Meat and Egg Production (5th ed. 2002) at page 783, attached herewith as **Exhibit D**.

[6] *See e.g.*, Abstract ("The present invention is directed to bacteriophage therapy…); col. 7, lines 51-52 ("The present invention can be applied across the spectrum of bacterial diseases…); col. 8, lines 4-5 ("The second embodiment of the present invention is the development of methods to treat bacterial infections…"); col. 8, lines 14-18 ("While it is contemplated that the present invention can be used to treat any bacterial infection in an animal, it is particularly contemplated that the methods described herein will be very useful as a therapy (adjunctive or stand-alone) in infections caused by drug-resistant bacteria.").

is directed only to "therapy" of animals and does not teach or suggest applying bacteriophage to "freshly-hatched" birds before transferring the birds to a chicken house, Merril does not teach or suggest each and every limitation of the claimed invention.[7]

Taylor does not cure the deficiencies of Merril. Indeed, Taylor does not teach or suggest the step of "applying at least one bacteriophage to at least one freshly-hatched bird before transferring said at least one freshly-hatched bird to a chicken house." Rather, Taylor is directed to treating eggs, not hatched birds.

Holzman also fails to cure the deficiencies of Merril. The Office Action states that Holzman "discloses using phages to target pathogens…as a way of potentially clearing the poultry yards of *S. enteriditis*." O.A. at page 6. Assuming Holzman discloses a method of using bacteriophages, Applicants submit that Holzman fails to disclose any specific method and/or steps for carrying out such a method of using bacteriophages. To be sure, Holzman does not teach or suggest a method of applying bacteriophage to a bird at a particular stage of life — after hatching and before transferring the bird to a chicken house — as required by the claimed invention. At best, Holzman is a generic reference that suggests applying bacteriophage for a variety reasons including at least therapy. This disclosure alone, or in combination with the cited references, is insufficient to render the claimed invention unpatentable because the claims are limited to a specific application of bacteriophage neither taught nor suggested by Holzman, Merril or Taylor.

Accordingly, Applicants submit that the references alone, or in combination, do not teach each and every claim limitation. Applicants also submit the Office Action does not provide a specific reason why one of ordinary skill in the art would be motivated to modify the method of Merril to apply "at least one bacteriophage to at least one freshly-hatched bird before transferring said at least one freshly-hatched bird to a chicken house."

Applicants further submit that the Office Action fails to establish a *prima facie* case with respect to claims 88 and 89. Claim 88 is directed to the method of claim 43, and further comprises the step of applying at least one bacteriophage to the surface of an egg prior to hatching of the bird. Claim 89 is directed to the method of claim 43, and further comprises the step of transferring said bird to a chicken house after applying the bacteriophage. The Office Action includes claims 88 and

[7] Applicants also note that Merril's recitation of "livestock" does not necessarily include "poultry" or "chicken." *See* **Exhibit A** (separately defining "livestock" and "poultry").

89 in the rejection, but fails to point out where these references teach or suggest the limitations of claims 88 and 89.

In view of the foregoing, Applicants respectfully request withdrawal of the 103(a) rejection over Merril, Taylor and Holzman.

CONCLUSION

In view of the foregoing, Applicants respectfully request an indication of allowance of all claims.

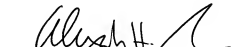
If the Examiner has any questions relating to this response, or the application in general, she is respectfully requested to contact the undersigned so that prosecution of this application may be expedited.

Concurrently with this paper, Applicants are filing a Request for Continued Examination that authorizes the Commissioner to charge **Deposit Account No. 50-0206** in the amount of $395.00 (small entity). Applicants believe that no additional fees are required for entry of this paper, but should any fees be required for entry and consideration of this amendment and response, the Commissioner is authorized to charge such fees to **Deposit Account No. 50-0206**.

Respectfully submitted,

HUNTON & WILLIAMS LLP

Dated: September 4, 2007 By:



Laurence H. Posorske, Ph.D.
Registration No. 34,698

Alexander H. Spiegler
Registration No. 56,625

HUNTON & WILLIAMS LLP
Intellectual Property Department
1900 K Street, N.W., Suite 1200
Washington, DC 20006-1109
(202) 955-1500 (telephone)
(202) 778-2201 (facsimile)

Exhibit A

TITLE 7--AGRICULTURE

CHAPTER 9--PACKERS AND STOCKYARDS

SUBCHAPTER I--GENERAL DEFINITIONS

Sec. 182. Definitions

When used in this chapter--

(1) The term ``person'' includes individuals, partnerships, corporations, and associations;

(2) The term ``Secretary'' means the Secretary of Agriculture;

(3) The term ``meat food products'' means all products and byproducts of the slaughtering and meat-packing industry--if edible;

(4) The term ``livestock'' means cattle, sheep, swine, horses, mules, or goats--whether live or dead;

(5) The term ``livestock products'' means all products and byproducts (other than meats and meat food products) of the slaughtering and meat-packing industry derived in whole or in part from livestock;

(6) The term ``poultry'' means chickens, turkeys, ducks, geese, and other domestic fowl;

(7) The term ``poultry product'' means any product or byproduct of the business of slaughtering poultry and processing poultry after slaughter;

(8) The term ``poultry grower'' means any person engaged in the business of raising and caring for live poultry for slaughter by another, whether the poultry is owned by such person or by another, but not an employee of the owner of such poultry;

(9) The term ``poultry growing arrangement'' means any growout contract, marketing agreement, or other arrangement under which a poultry grower raises and cares for live poultry for delivery, in accord with another's instructions, for slaughter;

(10) The term ``live poultry dealer'' means any person engaged in the business of obtaining live poultry by purchase or under a poultry growing arrangement for the purpose of either slaughtering it or selling it for slaughter by another, if poultry is obtained by such person in commerce, or if poultry obtained by such person is sold or shipped in commerce, or if poultry products from poultry obtained by such person are sold or shipped in commerce; and

(11) The term ``commerce'' means commerce between any State, Territory, or possession, or the District of Columbia, and any place outside thereof; or between points within the same State, Territory, or possession, or the District of Columbia, but through any place outside thereof; or within any Territory or possession, or the District of Columbia.

(12) Swine contractor.--The term ``swine contractor'' means any person engaged in the business of obtaining swine under a swine production contract for the purpose of slaughtering the swine or selling the swine for slaughter, if--

(A) the swine is obtained by the person in commerce; or

(B) the swine (including products from the swine) obtained

by the person is sold or shipped in commerce.

(13) Swine production contract.--The term ``swine production contract'' means any growout contract or other arrangement under which a swine production contract grower raises and cares for the swine in accordance with the instructions of another person.

(14) Swine production contract grower.--The term ``swine production contract grower'' means any person engaged in the business of raising and caring for swine in accordance with the instructions of another person.

(Aug. 15, 1921, ch. 64, title I, Sec. 2(a), 42 Stat. 159; Pub. L. 94-410, Sec. 3(c), Sept. 13, 1976, 90 Stat. 1249; Pub. L. 100-173, Sec. 2, Nov. 23, 1987, 101 Stat. 917; Pub. L. 107-171, title X, Sec. 10502(a), May 13, 2002, 116 Stat. 509.)

Codification

Section is composed of subsec. (a) of section 2 of act Aug. 15, 1921. Subsec. (b) of section 2 is classified to section 183 of this title.

Amendments

2002--Pars. (12) to (14). Pub. L. 107-171 added pars. (12) to (14).

1987--Pars. (6) to (11). Pub. L. 100-173 added pars. (6) to (10) and redesignated former par. (6) as (11).

1976--Pars. (4), (5). Pub. L. 94-410 substituted ``livestock'' for ``live stock'' in par. (4) and for ``live-stock'' in par. (5).

Effective Date of 1987 Amendment

Section 12 of Pub. L. 100-173 provided that: ``This Act and the amendments made by this Act [enacting sections 197 and 228b-1 to 228b-4 of this title, amending this section and sections 192, 209, 221, 223, 227, and 228a of this title, repealing sections 218 to 218d of this title, and enacting provisions set out as notes under sections 181 and 227 of this title] shall take effect 90 days after the date of the enactment of this Act [Nov. 23, 1987].''

Savings Provision

Section 10 of Pub. L. 94-410 provided that: ``Pending proceedings shall not be abated by reason of any provision of this Act [enacting sections 196 and 228a to 228c of this title and amending this section and sections 183, 191-193, 201, 204, 207, 209, 210, 212, 213, 228, and 229 of this title], but shall be disposed of pursuant to the provisions of the Packers and Stockyards Act, 1921, as amended [this chapter], and the Act of July 12, 1943 [section 204 of this title], in effect immediately prior to the effective date of this Act [Sept. 13, 1976].''

Exhibit B

Journal of Applied Bacteriology 1979, 47, 443–455 572/031/79

Effect of Ammonia Concentration on the Composition, Hydrolytic Activity and Nitrogen Metabolism of the Microbial Flora of the Rumen

R. J. WALLACE

Microbiology Department, Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, Scotland

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The mean NH_3 concentration in the rumen of sheep fed whole barley (0·8 kg/d) by continuous feeders was increased from 6·1 to 13·4 mM by supplementing the feed with urea (30 g/kg). This change caused a 90% increase in the rate of degradation of rolled barley, and smaller increases in the rates of degradation of protein and plant fibre in the rumen. The total viable count and numbers of pectinolytic bacteria in rumen fluid increased with the urea supplement. Enzyme studies indicated that NAD-linked glutamate dehydrogenase was the main pathway of NH_3 assimilation by rumen bacteria at both NH_3 concentrations. Glutamate was the main free amino acid found in the rumen at low NH_3 but, despite the low activity of alanine dehydrogenase and glutamate–pyruvate aminotransferase, alanine was the principal amino acid at high NH_3 concentrations. Hydrolytic rumen bacteria may require the higher NH_3 concentration either for effective NH_3 assimilation by an unknown mechanism involving alanine or for full expression of enzyme activity.

IN THE FORMULATION of diets for ruminants, it is important to optimize the balance between the nitrogen and energy content of the feed, so that a balanced rumen fermentation occurs and maximum voluntary intake and efficiency of feed utilization can be achieved (Roy *et al.* 1977). Urea, which is rapidly hydrolysed to ammonia and carbon dioxide in the rumen, is now used widely to increase the nitrogen content of ruminant feeds low in crude protein (see Chalupa 1972). Optimal levels of urea addition to different diets have not yet been established, however, largely because the effects of changing the ruminal NH_3 concentration on the rumen fermentation are not understood.

Satter & Slyter (1974) demonstrated that a mixed population of rumen bacteria could assimilate NH_3 from dilute solutions *in vitro*. Indeed subsequent nutritional models were based on the principle that urea supplementation should be adjusted to minimize accumulation of NH_3 in the rumen and thereby maximize the efficiency of N retention by the ruminant animal (Roffler & Satter 1975; Satter & Roffler 1977). In contrast, Mehrez *et al.* (1977) recommended a high ruminal NH_3 concentration for sheep fed a barley diet, as they found that a considerable excess of ammonia in the rumen increased the rate of degradation of the feed and so improved the efficiency with which constituents of the feed other than urea were used.

Some effects of NH_3 concentration on the nitrogen metabolism of rumen bacteria have been described for fermentations *in vitro* (Shimbayashi *et al.* 1975; Erfle *et al.* 1977). The object of the work described in this paper was to examine the effect of changing the ruminal NH_3 concentration on the nitrogen metabolism of rumen bacteria *in vivo*, and on the degradative functions of those bacteria, in order to assess the advantages afforded to the fermentation process by an apparent excess of NH_3 in the rumen of barley-fed sheep.

0021-8847/79/060443+13$01.00/0

Materials and Methods

Animals and diets

Two Suffolk × (Finnish Landrace × Dorset Poll) sheep (12–20 months of age and fitted with permanent rumen cannulae of 40 mm diam.) were used. Approximately 300 ml of rumen liquor from each animal was transferred to the other at the beginning of the experiment, so that it was possible for the same microbial population to establish in both animals. They had free access to water and received their diet from automatic feeders which supplied the daily ration (0·8 kg) continuously over a 24 h period. The diet consisted of whole barley (nitrogen content, 14·0 g/kg dry matter) fortified with vitamins and trace minerals (1·25 g/kg barley; Ørskov *et al.* 1972) and Na_2SO_4 (4 g/kg barley) by the absorption method of Ørskov *et al.* (1974). The sheep were fed this diet for 3–4 months, and then a diet of barley supplemented further by absorption of 30 g urea/kg barley for 2–3 months before being returned to the first diet. A four week period of adaptation to each diet was allowed before samples were taken for analysis. A third sheep of the same breed was fed a diet of dried grass and concentrate (2 : 1, 0·9 kg/d).

Sampling

Samples of rumen fluid were removed *via* the rumen cannula using a glass sampling tube (13 mm diam.). All samples except those for bacterial counts were immediately filtered through two layers of muslin. Samples for bacterial counts were taken at weekly intervals after the period of adaptation. Six samples were taken from sheep receiving whole barley without urea supplementation, and four samples were taken during the period of urea supplementation. Samples for analysis of pH, VFA, NH_3 and for microscopic examination were taken 2–3 times/week. Twelve samples were taken, at intervals of four days or more, for enzyme assays during each of the periods of barley feeding. Two similar samples, with an intervening period of four weeks, were taken from the grass-fed sheep. Amino acid analysis was done on two samples taken from each sheep during each period of barley feeding, the minimum period between each sample being two weeks.

Analytical methods

The N content of barley was determined by the automated Kjeldahl method (Davidson *et al.* 1970). The NH_3 concentration of filtered samples of rumen fluid, diluted to 0·1–0·5 mM, was assayed by the method of Weatherburn (1967). The final reaction mixture was centrifuged (3000 *g*/15 min/4 °C) before absorbance was determined. The protein content of cell-free extracts used in enzyme assays was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Volatile fatty acids were estimated by gas chromatography (Wallace 1978). TCA extracts of rumen fluid and of cell-free rumen liquor (prepared by centrifugation of filtered rumen fluid, 11 600 *g*/1 h/4 °C) were prepared for amino acid analysis by addition of 1 vol. 25% (w/v) TCA to 4 vol. of rumen liquor, centrifugation (11 600 *g*/15 min/4 °C), and three-fold extraction of the supernatant fluid with petroleum spirit (b.p. 40–60 °C). The extracted aqueous solution was evaporated to dryness and resuspended in citrate buffer (per litre: sodium citrate, 19·6 g; thiodiglycol, 5 ml, and octanoic acid, 0·1 ml all adjusted to pH 2·0 with HCl). The amino acid content of this suspension was analysed using a Locarte analyser modified to give tape output (Davidson *et al.* 1974). A sample

(0·25 ml) containing material from 1–4 ml rumen liquor and 0·1 μmol norleucine, was applied to the column. Occasionally it was necessary to remove insoluble material by centrifugation of the resuspended extract before analysis. Urea was determined by the method of Marsh *et al.* (1965) on TCA extracts neutralized with NaOH.

Measurement of degradation rates

The 'polyester bag' technique (Mehrez & Ørskov 1977) was used to determine the rate of degradation of barley and other substrates in the rumen. The method was modified so that freeze-drying was used in the procedure to obtain dry matter. The half-life ($t_{\frac{1}{2}}$) of the material under study was determined from the gradient of the exponential decay curve.

Bacterial counts

Anaerobic culture techniques (Hungate 1969) were used to count viable bacteria in whole rumen liquor. Ten-fold dilutions of liquor were prepared in the diluting fluid of Mann (1968). The total viable count and the number of amylolytic bacteria were assessed by the method of Hobson (1969) using medium 2 of Kurihara *et al.* (1968). Proteolytic bacteria were counted on a casein medium—similar to medium 7 of Hobson (1969)—containing (/100 ml): minerals (a), 15 ml; minerals (b), 15 ml; cysteine HCl, 0·05 g; casein, 1·0 g; clarified rumen fluid, 10 ml; tryptose, 0·3 g; agar, 2·0 g; resazurin, 0·1 mg; maltose, 0·5 g, and $NaHCO_3$, 0·4 g. The casein was autoclaved separately, and the maltose, cysteine and $NaHCO_3$ were added as a filter-sterilized solution. Numbers of cellulolytic bacteria were estimated by the filter paper strip method of Mann (1968), except that cellobiose was omitted from the medium. Lactobacilli were counted on SL medium (Rogosa *et al.* 1951) in Petri dishes. The medium for the quantitative isolation of xylan fermenters was medium 2 (Kurihara *et al.* 1968) in which starch was replaced by xylan (Cambrian Chemicals, Croydon CRO 4XB). Pectin (from citrus fruits, Sigma Chemical Co., Poole, Dorset BH17 7NH) replaced starch in medium 2 for counting pectinolytic bacteria. Zones of pectin hydrolysis were visualized by filling the roll tubes with 4% (w/v) cetyl trimethyl ammonium chloride which renders the pectin medium opaque apart from clear areas around pectinolytic colonies (Jayasankar & Graham 1970).

Films of rumen fluid were stained by Lillie's (1928) modification of Gram's method, counterstaining with carbol fuchsin. Protozoa were counted by the method of Hungate *et al.* (1971).

Enzyme activity

Filtered rumen liquor was centrifuged (11 600 g/15 min/4 °C), and the pellet resuspended to the same volume in 20 mM Tris (pH 7·0). The bacteria were disrupted by sonication (5 A/5 min/0 °C). As additional agitation with Ballotini beads did not release any additional enzyme activity, sonication alone was used. More than 90% of each enzyme activity was released into the medium by sonication. Whole cells were removed by centrifugation (11 600 g/1 h/4 °C), and the supernatant liquor was used immediately for enzyme assays or stored at −60 °C.

All enzyme assays were done at 25 °C. Glutamine synthetase (EC 6.3.1.2) was measured as the glutamyl–hydroxamate transferase by the method of Woolfolk *et al.* (1966). The principal amino acid dehydrogenases were assayed by methods similar to those of Meers *et al.* (1970), as were the glutamate synthases (Table 1). In experiments

TABLE 1

Reaction mixtures for measurement of NAD(P)-linked enzymes of NH_3 metabolism in sonicated rumen bacteria

Enzyme	Tris (pH 7·6)	Cofactor (0·25 μmol/ml)	μmol/ml				
			Na 2-oxoglutarate	Na pyruvate	Na oxalacetate	NH_4Cl*	Glutamine*
Glutamate dehydrogenase	33·3	NADH	5·0	—	—	160	—
Glutamate dehydrogenase	33·3	NADPH	5·0	—	—	40	—
Alanine dehydrogenase	33·3	NADH	—	5·0	—	160	—
Aspartate dehydrogenase	33·3	NADH	—	—	5·0	160	—
Glutamate synthase	33·3	NADH	5·0	—	—	—	5·0
Glutamate synthase	33·3	NADPH	5·0	—	—	—	5·0

* The reaction rate due to each enzyme was determined by the difference in the rate of NAD(P)H oxidation in the presence and in the absence of the *N* donor.

to test for other enzyme activities, the basic assays of Meers *et al.* (1970) were used, with α-oxo acids present at 5 mM, NAD(P)H at 0·25 mM and either NH_4Cl at 40 mM or amide at 5 mM. K_m values for ammonia and α-oxo acids were determined by conventional double reciprocal plots (Dixon & Webb 1964). The enzyme assays were done in a double-beam recording spectrophotometer (CE595; Cecil Instruments, Cambridge, England) and enzyme activity was measured by the difference between the initial rates of NAD(P)H oxidation in the complete reaction mixture and in the mixture with the N donor absent.

The spectrophotometric assay of glutamate–pyruvate aminotransferase (EC 2.6.1.2) used a modified version of the method of Bergmeyer & Bernt (1974) to overcome interference from the glutamate dehydrogenase present in sonicated rumen bacteria. The reaction mixture contained 100 mM phosphate buffer (pH 7·4), 0·25 mM NADPH, 6·7 mM sodium glutamate, 6·7 mM sodium pyruvate and 160 mM NH_4Cl. The 2-oxoglutarate formed from glutamate was converted back to glutamate by endogenous glutamate dehydrogenase at the expense of NADPH. The rate of NADPH oxidation in the presence of the amino-transferase inhibitor, aminooxyacetic acid (1·0 mM; Miflin & Lea 1975), was used as the reference reaction mixture.

Results

Supplementing whole barley with 3% (w/v) urea resulted in more than a doubling of the mean ruminal NH_3 concentration (Table 2). The pH of rumen fluid was reduced from 6·29 ± 0·06 to 6·13 ± 0·03 by the supplementation. The volatile fatty acid (VFA) concentrations were virtually unchanged and urea could not be detected in rumen fluid. The concentrations of acetate, propionate and butyrate at low NH_3 were 51·16 ± 1·46, 25·04 ± 1·30 and 13·51 ± 0·92 mM respectively, and 49·36 ± 1·28, 23·54 ± 1·12 and 12·99 ± 0·43 mM at high NH_3.

TABLE 2

Effect of urea supplementation on ruminal NH_3 concentration and on the rate of loss of dry matter from rolled barley, wheat gluten and wheat bran incubated in polyester bags suspended in the rumen

Diet	NH_3 (mM)	Half-life: Rolled barley* (h)	Half-life: Wheat gluten† (d)	Half-life: Wheat bran‡ (d)
Whole barley	6·13 ± 0·56§	9·28 ± 1·66	4·23 ± 0·22	7·84 ± 1·50
Whole barley + 3% urea	13·39 ± 0·84	4·89 ± 0·64	3·29 ± 0·76	5·77 ± 0·75

* The $t_{\frac{1}{2}}$ of barley was determined for the fraction degraded in a 30 h incubation (see text); † no gluten was lost from a polyester bag incubated in non-sterile 40 mM KH_2PO_4 buffer (pH 7·0), with stirring, at 4 °C for 16 h. It was therefore concluded that weight loss *in vivo* was due to the action of rumen bacteria. ‡ Wheat bran was washed several times in distilled water to remove adhering starch before being dried and used for incubations. § In this table, and those following, the error calculated is the standard error of the mean of a number of measurements made on different days.

Effect of ruminal NH_3 concentration on rates of degradation

Rolled barley suspended in the rumen in bags made of polyester material was used to estimate the rate of degradation of the feed (Mehrez & Ørskov 1977). The degradability of the barley was found to be 76·2%—estimated from 30 h incubations—at both NH_3 concentrations. As the degradation of this fraction was found to occur exponentially, the half-life of this 'rapidly-degraded' part of the feed could be determined. The rate of degradation at the higher NH_3 was 90% greater than that at the lower concentration (Table 2). Similar experiments using wheat gluten or wheat bran instead of rolled barley also showed increases in the rate of digestion at the higher NH_3 concentration, although neither rate was accelerated as much as that of rolled barley (Table 2).

Counts of different classes of rumen bacteria

There was considerable variation in the counts of the strictly anaerobic rumen bacteria. The total viable count was increased somewhat when the animals received barley supplemented with urea, although the numbers of bacteria of different functional groups varied (Table 3). The largest difference between the high and low NH_3 rumen fluid floras was found in the numbers of pectinolytic bacteria, which increased by more than an order of magnitude in sheep receiving urea-supplemented barley. Indeed, this was the only group of bacteria in which the range of counts obtained from the two dietary conditions did not overlap. As the counts of proteolytic and cellulolytic bacteria were so variable—satisfactory means could not be calculated—no conclusion could be made as to the effect of ruminal NH_3 concentration on the numbers.

Microscopic examination of Gram-stained films of rumen contents of the sheep receiving the different diets showed no discernible pattern. Variations in the numbers of different morphological types were seen to be large in samples taken from sheep on separate days, even on the same diet and in a single animal. The films prepared from the rumen contents of animals on both dietary conditions were considered to be typical or rumen fluid from sheep fed whole barley. *Bacteroides* was the main morphological type with variable numbers of Gram positive and negative small cocci occurring singly or in clumps of 2 or 4 bacteria. Gram negative small curved rods were always present. There were few Gram positive rods. Of the larger bacteria, large selenomonads and sarcina-like organisms were prevalent, with fluctuating numbers of chain-forming large Gram negative cocci. The protozoal population was similar in both animals following cross-inoculation, and was stable in number ($\approx 10^6$/ml), consisting mainly of *Entodinia*. Sheep receiving whole barley alone also had *Polyplastron* as 1–10% of the total protozoa. When urea was introduced, the number of this species fell to 0·1% of the total number in each sheep within 10 d and disappeared completely after a further week. No attempt was made to re-introduce *Polyplastron* and, as the animals were isolated, they had no opportunity to regain protozoa naturally.

Enzymes of NH_3 assimilation in rumen bacteria

Several enzymic reactions of the form

$$R_1\text{-CO-COOH} + R_2\text{-NH}_2 \xrightarrow{\text{NAD(P)H} \rightarrow \text{NAD(P)}} R_1\text{-CH(NH}_2\text{)-COOH} + R_2\text{-OH}$$

were measured in cell-free extracts of rumen bacteria. Where the amino group donor was ammonia ($R_2 = H$), the main enzymic activities were NAD- and NADP-linked

TABLE 3

Counts of bacteria in rumen fluid of sheep fed whole barley and whole barley + 3% urea

Diet	Total viable bacteria/ml	Hydrolytic bacteria/ml					Lactobacilli/ml
		Starch	Xylan	Pectin	Cellulose	Casein	
Whole barley	$2{\cdot}8 \pm 0{\cdot}7 \times 10^8$	$1{\cdot}9 \pm 0{\cdot}7 \times 10^8$	$1{\cdot}4 \pm 0{\cdot}6 \times 10^7$	$4{\cdot}9 \pm 1{\cdot}5 \times 10^6$	$10^7, 10^6, 10^7$, $10^6, 10^5$	$<10^2, <10^2, 2 \times 10^8$	$7{\cdot}9 \pm 4{\cdot}7 \times 10^7$
Whole barley + 3% urea	$5{\cdot}3 \pm 1{\cdot}8 \times 10^8$	$0{\cdot}3 \pm 0{\cdot}1 \times 10^8$	$1{\cdot}3 \pm 0{\cdot}2 \times 10^7$	$8{\cdot}3 \pm 5{\cdot}4 \times 10^7$	$10^6, 10^7$ $10^6, 10^7$	$<10^2, <10^2, 2 \times 10^7$ $10^6 < 10^5$ $10^6 < 10^5$	$1{\cdot}2 \pm 0{\cdot}6 \times 10^7$

TABLE 4

Enzymes of ammonia assimilation in cell-free sonicated extracts of rumen bacteria from sheep fed whole barley, whole barley + 3% urea, and dried grass + concentrate

Diet	NADP-linked Glutamate dehydrogenase*	NAD-linked Glutamate dehydrogenase*	NAD-linked Alanine dehydrogenase*	NAD-linked Aspartate dehydrogenase*	Glutamine synthetase†
Whole barley	0·073 ± 0·040	1·99 ± 0·43	0·034 ± 0·007	1·14 ± 0·64	0·0074 ± 0·0036
Whole barley + 3% urea	0·007 ± 0·001	1·96 ± 0·17	0·008 ± 0·001	1·46 ± 0·13	0·0067 ± 0·0010
Grass + concentrate	0·017, 0·017	0·90, 1·41	0·146, 0·104	—	0, 0·0024

* μmol NAD(P)H oxidized/mg protein/min; † μmol γ-glutamyl hydroxamate formed/mg protein/min.

glutamate dehydrogenase (GDH), NAD-linked alanine dehydrogenase and NAD-linked aspartate dehydrogenase (Table 4). No NADP-linked alanine dehydrogenase or NADP-linked aspartate dehydrogenase was found. With glutamine as amino donor (R_2 = HOOCCH(NH_2)CH_2CH_2CO–), very low rates of reductive amination of 2-oxoglutarate were found, coupled either to NADH or to NADPH oxidation. Although accurate values for these glutamate synthase activities were impossible to obtain due to the low activity and the high background level of NAD(P)H oxidation, it was estimated that the activity was at or below 0·01 μmol NAD(P)H oxidized/mg protein/min. No difference between the two dietary regimes was detectable. Neither pyruvate nor oxalacetate could replace 2-oxoglutarate as acceptor, and a reaction mixture containing oxalacetate and asparagine in place of 2-oxoglutarate and glutamine did not cause oxidation of either NADH or NADPH.

The specific activities of NAD-linked GDH and NAD-linked aspartate dehydrogenase were high and not altered by supplementing the diet with urea; the activities of NADP-linked GDH and NAD-linked alanine dehydrogenase were reduced markedly at the higher ruminal NH_3 concentration (Table 4). Glutamine synthetase activity was low in both cases (Table 4).

The enzymes present in rumen fluid of a sheep receiving a dried grass and concentrate diet were similar to those of the barley-fed animals. NAD-linked GDH again showed the highest activity, followed by alanine dehydrogenase and NADP-linked GDH (Table 4).

The K_m's for NH_3 of the four main amino acid dehydrogenases were determined from double reciprocal plots of the Lineweaver–Burk type with enzyme preparations obtained from both high and low NH_3 rumen fluid. All of the plots were linear and the calculated K_m's showed that the order of affinity of the enzymes for NH_3 was NADP-linked GDH > NAD-linked GDH > alanine dehydrogenase > aspartate dehydrogenase (Table 5). The K_m's of the acceptor α-oxo acids in the rumen preparations were all low (Table 5).

The spectrophotometric assay of glutamate–pyruvate aminotransferase in cell-free extracts of sonicated preparations was attempted by the method of Bergmeyer & Bernt (1974); the rate of pyruvate production from a mixture of 2-oxoglutarate and L-alanine was measured spectrophotometrically by the subsequent reduction of pyruvate to

TABLE 5

K_m for NH_4^+ and α-oxo acids of the enzymes of NH_3 assimilation in cell-free extracts of rumen bacteria obtained from sheep fed a barley diet

Substrate	NADP-linked Glutamate dehydrogenase	K_m (mM)* NAD-linked Glutamate dehydrogenase	Alanine dehydrogenase	Aspartate dehydrogenase
NH_4^+	1·8–3·1	20–30	70	250
α-oxo acid	0·2	3·1	4·4	0·7

* K_m's of enzymes for NH_3 were determined on cell-free extracts from both low and high NH_3 rumen fluid. No differences in K_m's were apparent between the two conditions.

lactate by NADH in the presence of lactate dehydrogenase. Glutamate dehydrogenase activity present already in sonicated rumen bacteria made a 'proper' control in this assay impossible to obtain. Thus aminotransferase activity was measured in the opposite direction, by the conversion of 2-oxoglutarate to glutamate using endogenous NADP-linked GDH. Activity was measured as the difference in the initial rate of NADPH oxidation between the complete reaction mixture, and another containing 1 mM aminooxyacetate, a specific inhibitor of aminotransferase activity (Miflin & Lea 1975). The glutamate-pyruvate aminotransferase activity measured in this way was 0·0066 μmol/mg protein/min in the rumen fluid of sheep receiving urea-supplemented barley. As this activity was unstable, it was measured in fresh samples immediately after sonication.

Pools of free amino acids in rumen fluid

The concentrations of amino acids in the acid-soluble fraction of rumen fluid were compared for the two dietary regimes (Table 6). As the separation of threonine, serine, asparagine and glutamine was poor, these amino acids have been considered as a single quantity in Table 6. Glutamate, with a mean concentration of almost 0·3 mM, was the main free amino acid in the samples of rumen fluid having low concentrations of NH_3. Aspartate was the second most abundant amino acid (0·07 mM); alanine, lysine, arginine and valine were present at >0·04 mM. No amino acid was absent, although the levels of methionine, *iso*leucine and leucine were very low. Proline and cysteine were present, but the analytical method could not measure their concentrations accurately.

The increase in ruminal NH_3 concentration on urea supplementation caused the glutamate pool to fall and the alanine pool to rise. There were few changes in the pools of the other amino acids (Table 6). Alanine replaced glutamate as the predominant amino acid at a concentration of 0·2 mM, with the glutamate pool falling to 0·13 mM. Analysis of the supernatant fluid of centrifuged rumen fluid showed that some of the free amino acids, such as glutamate and aspartate, existed mainly intracellularly, while others, such as the neutral amino acids, were mainly extracellular. The overall totals showed that 62% of the amino acids released by TCA extraction occurred intracellularly.

TABLE 6

Free amino acid pools in rumen fluid of sheep fed whole barley and whole barley + 3% urea

Amino acid	Concentration (nmol/ml)		
	Whole barley	Whole barley + 3% urea	Cell-free supernate*
Aspartate	71 ± 7	66 ± 5	15, 11
Serine/threonine/ glutamine/asparagine	65 ± 4	57 ± 12	0, 0
Glutamate	291 ± 50	132 ± 8	13, 0
Proline	present	present	0, 0
Glycine	31 ± 8	46 ± 8	36, 23
Alanine	59 ± 7	204 ± 11	93, 53
Cysteine	present	present	0, 0
Valine	42 ± 5	47 ± 12	64, 52
Methionine	3 ± 1	11 ± 2	0, 0
*Iso*leucine	8 ± 1	14 ± 3	15, 8
Leucine	7 ± 1	19 ± 5	21, 12
Tyrosine	11 ± 3	10 ± 2	20, 21
Phenylalanine	28 ± 6	19 ± 5	23, 20
Histidine	24 ± 10	16 ± 4	0, 0
Ornithine	12 ± 2	12 ± 1	0, 0
Lysine	48 ± 7	58 ± 11	36, 30
Arginine	42 ± 8	30 ± 5	present

* Measured in the supernatant fluid from an 11 600 g centrifugation (1 h) of two samples of filtered rumen fluid from sheep fed whole barley + 3% urea.

The most abundant ninhydrin-reacting peak in the elution profile, apart from NH_3, occurred immediately following the NH_3 peak, in a position which did not correspond to any of the amino acids. As this compound was steam-volatile in alkaline solution—as was NH_3—, eluted from the column in the same position after distillation, and in the same position as authentic methylamine, it was concluded that the compound was methylamine. The concentration of this amine was variable, but in a manner which did not correspond with the NH_3 concentration, or any other demonstrable factor, and was often as high as 0·45 mM. Removal of bacteria by centrifuging did not cause the loss of any methylamine from the fluid. The concentrations of free glutamate and alanine in the rumen of sheep receiving a grass and concentrate diet, were 0·170 mM and 0·122 mM respectively, at a ruminal NH_3 concentration of 16·1 mM.

Discussion

As found by Mehrez *et al.* (1977), the hydrolytic activity of rumen bacteria of barley-fed sheep increased when the concentration of NH_3 in the fluid was increased to a level considered by some workers (e.g. Satter & Slyter 1974) to be in excess of the requirements of rumen bacteria (Table 2). An increase in the number of viable bacteria in the rumen accompanied this change in activity, but it was not clear which particular types of hydrolytic bacteria were involved, as only pectinolytic bacteria showed detectable increase in numbers (Table 3), an activity which does not limit the rate of fermentation in the rumen (Howard 1961).

1 whole barley

free supernate*

15, 11
0, 0

13, 0
0, 0
36, 23
93, 53
0, 0
64, 52
0, 0
15, 8
21, 12
20, 21
23, 20
0, 0
0, 0
36, 30
present

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The discovery of glutamate synthase in rumen bacteria at low NH_3 concentrations (Erfle *et al.* 1977) indicated that, as in several other bacteria, NH_3 uptake might be ATP-dependent at low NH_3 concentrations, but not at higher concentrations, and that molar growth yields might therefore be affected by NH_3 concentration (Brown *et al.* 1974). Very little glutamate synthase, however, and only low levels of glutamine synthetase activity were present (Table 4). Other possible ATP-dependent systems for NH_3 uptake were not measured but as carbamyl phosphokinase activity is low in rumen bacteria (Chalupa *et al.* 1970) and as no enzyme analogous to glutamate synthase with asparagine as amide donor—which could be coupled to asparagine synthetase, as found in *Streptococcus bovis* (Burchall *et al.* 1964)—was demonstrable, it was concluded that energy-dependent NH_3 uptake was not of importance at either NH_3 concentration.

The activities (Table 4) and kinetic properties (Table 5) of the amino acid dehydrogenases implied that at both NH_3 concentrations glutamate dehydrogenase was likely to be the principal mechanism for NH_3 uptake in rumen bacteria, a conclusion also made by Hoshino *et al.* (1966), Palmquist & Baldwin (1966), Chalupa *et al.* (1970) and Erfle *et al.* (1977). Yet, as found in fermentations *in vitro* (Shimbayashi *et al.* 1975; Erfle *et al.* 1977) alanine was the predominant free amino acid at the higher NH_3 concentration (Table 6), despite the low activity of alanine dehydrogenase (Table 4) and glutamate-pyruvate aminotransferase, and the high K_m for NH_3 of the former enzyme (Table 5). Presumably alanine is synthesized under these conditions by an enzyme linked to a cofactor other than a pyridine nucleotide, although this has not been investigated. NAD-linked alanine dehydrogenase would appear to be of greater importance in sheep on a grass diet (Table 4), especially if accumulation of NH_3 occurs, as it does in *Escherichia coli* (Stevenson & Silver 1977), to overcome the high K_m (Table 5). It may be that the effect of NH_3 concentration on amino acid metabolism is related to the improved hydrolytic activity at high NH_3, if bacteria which assimilate NH_3 by the alanine pathway require a higher NH_3 concentration for growth and if the same bacteria are responsible for the degradation of plant material.

Methylamine has been found previously in rumen contents, although its metabolism is poorly understood (Hill & Mangan 1964). In barley-fed sheep, methylamine was the most abundant ninhydrin-reacting compound other than NH_3. As it occurred extracellularly, and its concentration did not change with NH_3 concentration, it is unlikely that this amine was in any way connected with the influence of NH_3 concentration on the rumen fermentation.

In conclusion, these experiments show that an increased NH_3 concentration in the rumen increased the size of the microbial flora and its hydrolytic activity. A change in NH_3 metabolism accompanied these changes, such that the synthesis of alanine assumed greater importance at high NH_3. A factor which was not investigated is the possible direct effect of NH_3 on the expression of enzyme activity. In view of the effect of NH_3 concentration on the urease activity of rumen bacteria (Wallace *et al.* 1979), this is a possibility which should be examined further.

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Exhibit C

Microflora Ecology of the Chicken Intestine Using 16S Ribosomal DNA Primers

E. Amit-Romach, D. Sklan, and Z. Uni[1]

The Faculty of Agricultural, Food and Environmental Quality Sciences, Hebrew University of Jerusalem, PO Box 12, Rehovot, 76100, Israel

ABSTRACT The microflora in the gastrointestinal tract of broiler chickens influences digestion, health, and well-being. Analysis of chicken gut microflora has been mainly by culture-based methods. Studies using these techniques have been useful for identification and analysis of specific groups of bacteria, however, the use of enrichment medium precludes even relative quantitation of bacterial species. Recent advances in ribosomal DNA-based molecular techniques make it possible to identify different bacterial populations in environmental samples without cultivation. In this study, the intestinal microflora was examined using 16S ribosomal DNA (rDNA) targeted probes from bacterial DNA isolated from intestinal and cecal contents of chickens at 4, 14, and 25 d of age. The ribosomal gene sequence was amplified using PCR with universal primers to determine total bacterial DNA and specific primers directed at 6 bacterial species: *Lactobacillus, Bifidobacterium, Salmonella, Campylobacter, Escherichia coli,* and *Clostridium*. The use of universal primers extends these methods to allow determination of relative proportions of different bacterial species.

The results indicated that in young chicks the major species present in the small intestines and ceca was *Lactobacilli*, with a *Bifidobacteria* population becoming more dominant in the ceca at older age. *Clostridium* was detected in some segments of the small intestine in young chicks. In older chickens, *Salmonella, Campylobacter,* and *E. coli* species were found in the ceca. This study has demonstrated the use of molecular techniques for determining relative proportions of bacterial species and monitoring pathogens in the chick gastrointestinal tract.

(*Key words*: bacteria, cecum, chicken, intestine, microflora)

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INTRODUCTION

The gastrointestinal tract is the major digestive and absorbing organ and it plays a crucial role in chicken growth. A diverse microbiota is found throughout the tract and is most extensive in the cecum (Barnes, 1972; Barnes et al., 1972; Mead and Adams, 1975; Mead, 1997). This microflora has a role in nutrition, detoxification of certain compounds, growth performance, and protection against pathogenic bacteria. The gut microflora influences health and well-being of host animals (Nurmi and Rantala, 1973; Mead, 1997; Vispo and Karasov, 1997; van der Wielen et al., 2002).

In poultry, the absence of normal microflora in the cecum has been considered a major factor in the susceptibility of chicks to bacterial infection (Barrow, 1992). Although the alimentary tract of the newly hatched chick is usually sterile, organisms rapidly gain access from the mother and the surrounding environment. Previous studies have documented the changes in microflora during the posthatch period. Large numbers of anaerobic bacteria capable of decomposing uric acid comprise the cecal flora of chicks 3 to 6 h after hatching (Mead and Adams, 1975). During the first 2 to 4 d posthatch, streptococci and enterobacteria colonize the small intestine and cecum. After the first week, lactobacillus predominate in the small intestine, and the cecum is colonized mainly by anaerobes (*Escherichia coli* and Bacteroides) with lower numbers of facultative aerobes (Lev and Briggs, 1956; Mead and Adams, 1975).

A typical microflora of adult birds in the small intestine is established within 2 wk, however, it was found that the adult cecal flora, which was mainly obligate anaerobes, took up to 30 d to develop. At that age, bifidobacteria and bacteroides predominate (Barnes et al., 1972).

Until recently, intestinal microflora were analyzed by culture-based methods (Barrow, 1992), and microbial ecologists relied largely on techniques requiring the growing of organisms on selective media. Several limitations are associated with culture-based approaches, par-

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[1]To whom correspondence should be addressed: uni@agri.huji.ac.il.

Abbreviation Key: rDNA = ribosomal DNA; rRNA = ribosomal RNA.

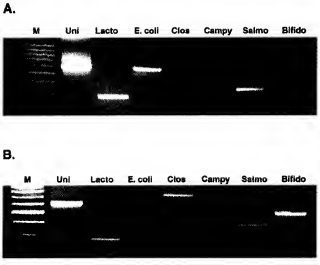

FIGURE 1. PCR products from 2 different animals (A. and B.) in a 2% agarose gel for each of the 6-primer sets that identify 6 bacterial groups (lanes 2 to 7) and the universal primers (lane 1). M = DNA size marker; Uni = universal primer (611 bp); Lacto = *Lactobacillus* (286 bp); E. coli = *Escherichia coli* (585 bp); Clos = *Clostridium* (722 bp); Campy = *Campylobacter* (857 bp); Salmo = *Salmonella* (396 bp); Bifido = *Bifidobacterium* (510 bp).

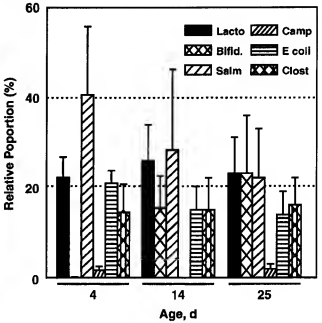

FIGURE 2. The proportion of PCR products of *Lactobacillus, Escherichia coli, Clostridium, Campylobacter, Salmonella,* and *Bifidobacterium,* relative to the universal PCR product calculated as percentage of the total examined bacteria in the chicken ceca with age. Lacto = *Lactobacillus;* Bifido = *Bifidobacterium;* Salm = *Salmonella;* Camp = *Campylobacter;* E. coli = *Escherichia coli;* Clost = Clostridium.

ticularly for surveying the intestinal ecosystem (Langendijk et al., 1995). In addition to being time and labor intensive, the use of selective media specific for different types of bacteria imposes an a priori bias. Various attempts have been made to determine the composition of the cecal microbiota in poultry, but the isolation methods used have not always been suitable for the oxygen-sensitive anaerobes, many of which are difficult to isolate and maintain (Mead, 1997).

In contrast, the recent development of PCR techniques has allowed the rapid and specific detection of a wide range of bacteria and should become a key procedure for detecting microorganisms. For many years, sequencing of the 16S ribosomal RNA (rRNA) gene has served as an important tool for determining phylogenetic relationships between bacteria. The features of this molecular target that make it a useful phylogenetic tool also make it useful for bacterial detection and identification in the clinical laboratory. Several studies have shown that sequence identification is useful for slow-growing, unusual, and fastidious bacteria, as well as for bacteria that are poorly differentiated by conventional methods (Patel, 2001). Recent advances in ribosomal RNA- and DNA-based molecular techniques make it possible to identify different bacterial populations in environmental samples without cultivation (Harmsen et al., 2000). This PCR methodology has been used to determine variation in bacterial population in the human colon and feces (Langendijk et al., 1995; Wang et al., 1996; Franks et al., 1998; Harmsen et al., 2000), the bovine rumen (Nelson et al., 1998), and the chicken cecum (Gong et al., 2002; Zhu et al., 2002). Previous reports have indicated that there is good correlation between PCR-based techniques and culture methods for species growing in cultures without significant enrichment (Wang et al., 1996).

There is a great importance in examining and monitoring the intestinal microflora because many bacterial species with pathogenicity toward humans have been found in the gastrointestinal tract of chickens and can thus be introduced into the food chain (Reeves et al., 1989; Davies and Wray, 1996; Brandt et al., 1999; Moreno et al., 2001).

The objectives of this study were to use PCR-based methods for detecting and quantifying, in the different parts of the intestine, the 16S ribosomal DNA (rDNA) of 6 bacterial species, *Lactobacillus, Bifidobacterium, Salmonella, Campylobacter, E. coli,* and *Clostridium,* which have a major role in chicken performance and consumer health, and to monitor the relative changes in the microbial population in the cecum and small intestine with age.

MATERIALS AND METHODS

Collection of Microbial Samples and DNA Isolation

Fifty Cobb chickens were grown from hatching for 25 d on wood shavings and were fed a standard commercial diet.[2] At 4, 14, and 25 d of age, 8 chickens were randomly selected and killed by cervical dislocation. The intestine and cecum were removed and treated as described by

[2]Matmor Feedmill, D. N. Evtach, Israel.

TABLE 1. PCR primers used in the study

Bacterial group	Primers	Sequence (5'-3')	Length (bp)	References
Universal	Unibac-f	CGTGCCAGCCGCGGTAATACG	611	
	Unibac-r	GGGTTGCGCTCGTTGCGGGACTTAACCCAACAT		
Lactobacillus	LAA-f	CATCCAGTGCAAACCTAAGAG	286	Wang et al., 1996
	LAA-r	GATCCGCTTGCCTTCGCA		
Escherichia coli	ECO-f	GACCTCGGTTTAGTCACAGA	585	Candrian et al., 1991; Wang et al., 1996
	ECO-r	CACACGCTGACGCTGACCA		
Clostridium	Clos58-f	AAAGGAAGATTAATACCGCATAA	722	
	Clos780-r	ATCTTGCGACCGTACTCCCC		
Campylobacter	Camp-f	ATCTAATGGCTTAACCATTAAAC	857	Denis et al., 2001
	Camp-r	GGACGGTAACTAGTTTAGTATT		
Salmonella	Sal201-f	CGGGCCTCTTGCCATCAGGTG	396	
	Sal597-r	CACATCCGACTTGACAGACCG		
Bifidobacterium	Bif164-f	GGGTGGTAATGCCGGATG	510	Langendijk et al., 1995
	Bif662-r	CCACCGTTACACCGGGAA		

Zhu et al. (2002). The contents of each segment (duodenum, jejunum, ileum, and cecum) were inverted into a sterile 15-mL tube containing 9 mL of sterile PBS, and homogenized by vortexing with glass beads (4-mm diameter) for 3 min. Debris was removed by centrifugation at 700 × *g* for 1 min, and the supernatant was collected and centrifuged at 12,000 × *g* for 5 min. The pellet was washed twice with PBS and stored at −20°C until DNA extraction. For DNA purification, the pellet was resuspended in EDTA and treated with lysosyme[3] (final concentration of 10 mg/mL) for 45 min at 37°C. The bacterial genomic DNA was isolated with the Wizard Genomic DNA purification kit.[4] DNA concentration and purity was checked spectrophotometrically.

All procedures were approved by the Animal Care and Welfare Committee of our Institute.

Primer Design and PCR Amplification of Bacterial 16s rDNA

Primers for *Lactobacillus, Clostridium, Campylobacter, Salmonella,* and *Bifidobacterium* were designed using the16S rRNA region for each bacterial group. Potential primer targets for *Lactobacillus, E. coli, Clostridium, Campylobacter, Salmonella,* and *Bifidobacterium* were identified by comparing the complete 16S rRNA sequences of bacterial groups using the programs BLAST, Seqweb, and RDPII (http://www.ncbi.nlm.nih.gov/BLAST/, http://seqweb.huji.ac.il/gcg-bin/seqweb.cgi, http://rdp.cme.msu.edu/html/). The target sites for the primers were identified as sequences that are invariant, or nearly so, in all members of a particular bacteria group, but differ significantly from all the representatives of the other 5 groups. The GenBank program BLAST was used to ensure that the proposed primers were complementary with the target species but not with other bacterial groups.

The primers used in this study are shown in Table 1. Universal primers identifying all known bacteria were designed using the invariant region in the 16s rDNA of the bacteria. The universal primer set was used for determining the total microflora population. Primers targeting *Lactobacillus* species were designed according to Wang et al. (1996), and primers for *E. coli* were modified from Candrian et al. (1991) by deleting 4 to 6 bases at the 5' end to fit our PCR conditions. The primer set for *Clostridium* was designed from 58bp and 780bp in the rDNA sequence (GenBank accession # AF316589). The primer set for *Campylobacter* species was according to Denis et al., (2001). Primers targeting *Salmonella* species were designed from the 201bp region and the 597bp region of the rDNA sequence (GenBank accession # AF332600), and the *Bifidobacterium* primer set was as described by Langendijk et al. (1995). For validation, each primer set was tested with known bacterial cultures.

For PCR amplification of the bacterial targets from intestinal contents, 5 μL of DNA extract was added to 45 μL of PCR mixture containing 27.5 μL of nuclease-free water, 5 μL of each primer, 1.5 μL of nucleotide (dNTP) mix, 5 μL of PCR buffer, and 1 μL of Taq polymerase. The PCR was conducted in a DNA Thermal Cycler.[5] The amplification conditions were: 1 cycle of 94°C for 3 min, 35 cycles of 94°C for 30 s, 60°C for 1 min and 68°C for 2 min, and finally 1 cycle of 68°C for 7 min. The PCR reaction was run with different numbers of cycles (25, 30, 35, 40, 45, or 50) for each primer set and 35 cycles was in the center of the exponential increase in PCR products. Products of PCR were visualized by agarose gel (2%) electrophoresis containing ethidium bromide.

Figure 1 shows the PCR products obtained from 16S rDNA for each of the 6 bacterial groups tested together with the universal primers in a representative sample from the chicken cecum. Background subtraction of gel images was performed and densitometric evaluation of the different bands was done with Gel-Pro Analyzer.[6]

The evaluation of the different PCR products was normalized to the density of the PCR product of the universal primers by densitometer scanning and was exhibited as arbitrary units (AU). To evaluate the relative proportion of each examined bacteria, all products were expressed relative to the content of the universal primer product and proportions of each bacterial group are presented where the total of the examined bacteria was set at 100%.

[3]Sigma Aldrich Co., St. Louis, MO.
[4]Promega Corporation, Madison, WI.
[5]MJ Research Inc., Waltham, MA.
[6]Media Cybernetics, L.P., Silver Spring, MD.

Results are presented as means ± SE and data were examined by the GLM procedure of SAS (SAS Institute, 1986) after arcsin transformation to test for significance, and returned to the original scale.

RESULTS

Microbial Distribution in Chicken Ceca at 4, 14, and 25 d

Analysis of chicks at 3 different ages (Figure 2), showed different relative proportions of the bacteria examined. In chicken ceca at 4 d, the relative proportion of *Lactobacilli* was about 25% of the total examined bacteria and *Bifidobacterium* was not detected. Relatively high proportions of *Salmonella* were detected (40%) and *Campylobacter* was present in minor amounts (2%). Almost one-third of the bacteria in the chicken ceca at this age consisted of *E. coli* and *Clostridium* species.

At the age of 14 d (Figure 2), the relative proportion of *Lactobacilli* and *Bifidobacterium* increased and reached 40% of the total bacteria. In contrast, the relative proportion of *Salmonella* was reduced by approximately 10%. *Campylobacter* was present only in trace amounts and proportions of *E. coli* and *Clostridium* changed little. At 25 d of age, almost one-half of the bacteria in the chicken ceca were *Lactobacilli* and *Bifidobacterium* species. Furthermore, the relative proportion of *Salmonella* had decreased by approximately 50% compared with that at 4 d. Proportions of *Campylobacter* remained small, whereas proportions of *E. coli* and *Clostridium* remained approximately 30%.

Microbial Diversity Along the Digestive Tract

Analysis of the microbial luminal contents of the different small intestine sites examined indicated that among the 6 examined bacterial species, only *Lactobacillus* was consistently detected in all intestinal regions (Figure 3). The results indicated that at d 4, most of the bacterial species were not detectable in the small intestines. Proportions of *Lactobacilli* changed little along the small intestines at a young age (Figures 3A and 4A). However, at d 25, the posterior segments exhibited lower levels of *Lactobacilli* compared with the anterior segment (Figure 4B). In addition, at d 25 *E. coli* and *Clostridium* were detected in the duodenum and ileum (Figure 3B).

DISCUSSION

Molecular techniques were used in this study to follow the ontogeny of the microbial populations in the small intestines and ceca of broiler chicks and to monitor the presence of harmful species.

In this study, *Lactobacillus* was the major species present in the duodenum of young chicks. Some clostridia species were found in the jejunum and ileum, as has been described previously in older chicks using culture methods

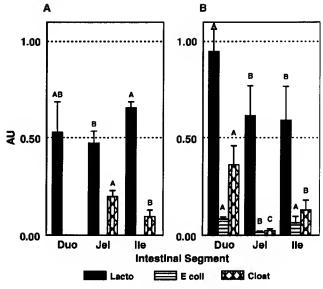

FIGURE 3. The proportions of bacterial populations in the content of the small intestines [duodenum (Duo), jejunum (Jej), and ileum (Ile)] at 4 d of age (A) and at 25 d of age (B). The evaluation of the different PCR products was normalized to the density of the PCR product of the universal primers by densitometer scanning and exhibited as arbitrary units (AU). Columns, for each bacteria, with different letters differ significantly among the intestinal regions ($P < 0.05$).

(Lev and Briggs, 1956; Barnes et al., 1972). The population in the cecum was more varied, with some *Salmonella* and *E. coli* species occurring, as has been previously observed using culture and molecular methods (Mead and Adams, 1975; Zhu et al., 2002). With age, the small intestine bacterial population remained predominantly lactobacilli, whereas in the cecum, *Bifidobacteria* began to develop and reached a stable proportion between 14 and 25 d. These results were again similar to previous reports that used culture methods (Barnes et al., 1972; Mead and Adams, 1975). In addition, it was demonstrated in this study that

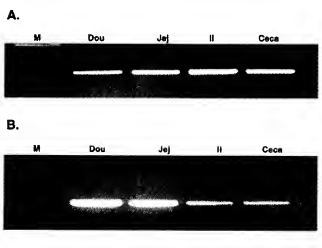

FIGURE 4. PCR products, in a 2% agarose gel, from representative chickens (A) 4 d old and (B) 25 d old, exhibiting *Lactobacillus* presence in different regions of the gastrointestinal tract. Lanes: M = size marker; Duo = duodenum; Jej = jejunum; Il = ileum; Caeca = cecum.

Salmonella and *Campylobacter* were present in some of the cecal samples.

In our study, we focused on 6 bacterial groups, some representing beneficial bacteria, (*Lactobacillus, Bifidobacteria*), some bacterial species potentially pathogenic to humans (*Salmonella, Campylobacter*), and some bacterial species possibly harmful to the chick (*E. coli, Clostridium*). *Lactobacillus* and *Bifidobacteria* are considered to be bacteria that stimulate growth and activity of other health-promoting bacteria and have been termed probiotic (Lucchini et al., 1998; Mikkelsen et al., 2003). *Salmonella* is a pathogen that causes gastroenteropathy in humans, has a broad distribution throughout the natural world, and a widespread occurrence in food animals, which may introduce this pathogen to the food chain (Davies and Wray, 1996; Reeves et al., 1989).

Thermotolerant *Campylobacter* is a common human enteric pathogen, which causes acute bacterial diarrhea worldwide, and which often originates from chick gut microflora (Moreno et al., 2001). Species of *Clostridium*, including *C. perfringens*, are widely distributed in the environment, inhabiting both human and animal gastrointestinal tracts (Brandt et al., 1999). *Escherichia coli* is an adaptive species that is both a commensal resident of the intestine and a versatile pathogen of humans and other animals, causing enteric infections and particular pathologies in different animal species (Dozois et al., 2003). Therefore, for animal and human health, there is great importance in developing a method that will enable accurate and rapid identification of the above bacteria. In the present report, we assumed that the universal primers are incorporated to all bacteria and thus could be used to quantitate the amounts of the different species. However, we restricted the analysis to detection of bacterial species without identification of specific subspecies including pathogens.

PCR-based techniques targeting the bacterial rDNA have been used to identify different bacterial species in fecal samples (Langendijk et al., 1995; Wang et al., 1996; Franks et al., 1998; Harmsen et al., 2000) However, the simultaneous use of universal primers directed at overall bacteria DNA has allowed us to extend this technique to estimate the relative proportions of different species present in the intestinal lumen. Some studies have used changes in the proportions of guanine plus cytosine to evaluate microbial populations (Apajalahti et al., 1998; Apajalahti et al., 2001). However, although this approach made it is possible to identify specific subgroups, it was not possible to determine species of bacteria in a mixed community and thus this method is less exact than the molecular identifications used here.

The use of molecular techniques has several advantages compared with the classical culture methods for enumerating bacteria, and does not introduce the bias of traditional methods. One major advantage is the rapidity and sensitivity of the determination compared with culture methods. Perhaps the most serious drawback of the culture-based methods is that only a small fraction of bacteria can be found; up to 99% of the bacteria in many environments fail to grow under artificial conditions (Amann et al., 1995; Hanson and Henson, 1996; Holben et al., 1998). This low recovery is due to the fact that growth requirements of most bacteria are still unknown and cannot be reproduced under laboratory conditions.

The results of this study demonstrate the application of PCR-based 16S rDNA techniques to determine changes in the microbiota in the chicken small intestines and ceca with age and to monitor the presence of potentially hazardous bacteria.

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Exhibit D

Commercial Chicken Meat and Egg Production

Fifth Edition

Edited by

DONALD D. BELL (emeritus)
Poultry Specialist
University of California
Riverside, California

WILLIAM D. WEAVER, JR.
Professor Emeritus
Department of Poultry Science
Virginia Tech
Blacksburg, Virginia
and
Department Head (retired)
Department of Poultry Science
Pennsylvania State University
University Park, Pennsylvania

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40
Operating the Hatchery

by Joseph M. Mauldin

The task of operating a commercial chicken hatchery requires continuous monitoring of numerous environmental factors that may affect chick hatchability and quality. Any small deviation in operating procedures, the quality of hatching eggs, and the care the chicks receive following the hatch can have a major impact on the success of the hatchery and, therefore, the economics of the operation. Not only are the chicks affected at the time, but oftentimes mismanagement in the hatchery can affect the overall performance of the flock.

40-A. SECURING HATCHING EGGS

In the United States and to a lesser extent in other countries, the poultry industry is vertically integrated. With *vertical integration*, the company that owns the hatchery generally owns the breeders supplying hatching eggs. However, in many countries where vertical integration is less prevalent, the hatchery does not have a guaranteed source of hatching eggs, and therefore must purchase from suppliers. In these situations, the quality of hatching eggs can vary with each supplier due to breeder flock age, management, fertility, holding and shipping times, sanitation, egg handling, and many other factors.

Source of Eggs

Hatchery owns the breeder hens. The most common situation in the United States is that the hatching eggs are produced by contract growers who have an agreement with the hatchery to supply eggs. Growers

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Calculating Hatchability

The only chicks that should enter into calculations for hatchability are first quality chicks. For each flock, the number of first quality chicks is divided by the number of total eggs set and then expressed as percentage hatchability. Hatcheries that sell second-class chicks calculate percentage hatchability for two categories: hatchability of first quality class and hatchability of second quality chicks. Most hatcheries place two or more additional (extra) chicks in each box to compensate for counting errors and for mortality that may occur in shipment.

40-D. CHICK DELIVERY

Safe and sanitary delivery of day-old chicks is the last of the many hatchery operations. Most deliveries are made by chick trucks or buses, although other means of transportation such as rail and air are sometimes used.

Chick Trucks and Buses

Specialized trucks and buses are used for delivery and are most often either custom-built or secured from a chick transport vehicle manufacturer. They must have good ventilation and a means of stacking and separating the boxes. Typical chick trucks can hold from 10,000 to 50,000 chicks. The size of chick trucks is determined by the size of the hatchery, delivery distance, and the number of days per week the hatchery operates.

Chick trucks need to have adequate heating, cooling, and ventilation systems to prevent the chicks from becoming stressed during delivery. Several thousand chicks generate a lot of heat and when confined into the relatively small space in the chick truck, adequate ventilation is required to remove the heat. Special fans, air intakes, and exhausts must be provided.

In cold climates, the chick compartments need to be heated. Water heated by the truck engine is the usual source of heat, while electric heaters are sometimes used. During summer months, or in hot climates, a refrigeration unit is required to prevent heat stress. In many cases, the cooling units are powered by small gasoline engines that must be properly exhausted.

The distance chicks are transported will determine the amount of truck cooling required. If the haul is only a short distance and can be completed during the cooler part of the day, adequate ventilation, rather than refrigeration, may be all that is needed.